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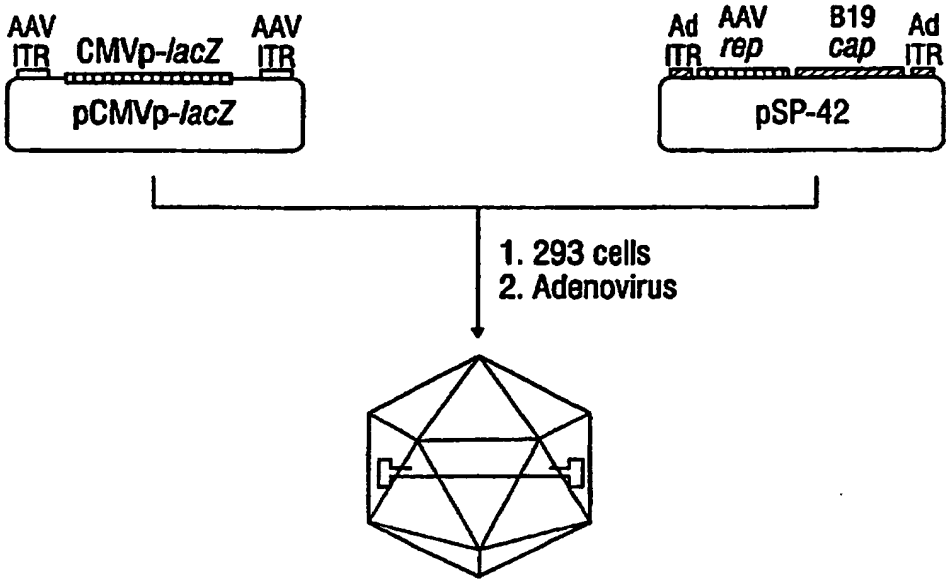

(57) Abstract

The present invention relates to the engineering, propagation and use of chimeric parvovirus vectors. These vectors, using sequences from adeno-associated virus (AAV) and the B19 may be used to deliver genes to various target cells, including those of erythroid lineage.

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DESCRIPTION

CHIMERIC PARVOVIRUS-BASED RECOMBINANT VECTOR SYSTEM THAT SPECIFICALLY TARGETS THE ERYTHROID LINEAGE

BACKGROUND OF THE INVENTION

The present application is a continuation–in–part of co–pending U.S. Provisional Patent Application Serial No. 60/061,364 filed October 8, 1997. The entire text of the above–referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of virology and molecular biology. More particularly, it concerns the engineering, propagation and use of viral vectors in the delivery of exogenous genes to cells.

2. Description of Related Art

Gene therapy protocols involving recombinant viral vectors are gaining wide attention and have immense potential to become the future mode of molecular medicine. Of the different viral vectors utilized to facilitate gene transfer, the retrovirus and adenovirus based vector systems have been extensively investigated over a decade. Recently, adeno-associated virus (AAV) has emerged as a potential alternative to the more commonly used retroviral and adenoviral vectors. While studies with retroviral- and adenoviral-mediated gene transfer raise concerns over potential oncogenic properties of the former, and immunogenic problems associated with the latter, AAV has not been associated with any such pathological indications.

In addition, AAV possesses several unique features that makes it more desirable than the other vectors. Unlike retroviruses, AAV can infect non-dividing cells; wild-type AAV has been characterized by integration, in a site-specific manner, into chromosome 19 of human cells (Kotin and Berns, 1989; Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991); and AAV also possesses anti-oncogenic properties (Ostrove *et al.*, 1981; Berns and Giraud, 1996). Recombinant AAV genomes are constructed by molecularly cloning DNA sequences of interest between the AAV ITRs, eliminating the entire coding sequences of the wild-type AAV

genome. The AAV vectors thus produced lack any of the coding sequences of wild-type AAV, yet retain the property of stable chromosomal integration and expression of the recombinant genes upon transduction both *in vitro* and *in vivo* (Berns, 1990; Berns and Bohensky, 1987; Bertran *et al.*, 1996; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997a).

Until recently, AAV was believed to infect almost all cell types, and even cross species barriers. However, it now has been determined that AAV infection is receptor-mediated (Ponnazhagan *et al.*, 1996; Summerford and Samulski, 1998). The B19 parvovirus, on the other hand, is a pathogenic virus and is an etiologic agent for a variety of human diseases (Anderson *et al.*, 1983; Brown *et al.*, 1984; Saarinen *et al.*, 1986; Serjeant *et al.*, 1981). In addition, B19 is known to infect only cells of erythroid lineage (Ozawa *et al.*, 1986; Ozawa *et al.*, 1987; Srivastava and Lu, 1988; Srivastava *et al.*, 1990). It recently has been determined that erythrocyte P antigen functions as the receptor for B19 infection in target cells.

There are many applications where targeting of erythroid tissues would be desirable. For example, where one seeks to produce proteins *in vitro*, it often is better to synthesize such proteins in the tissue in which they are normally produced to take advantage of correct post-translational processing, promoter specificity, *etc.* *In vivo* applications also are desired where genes are targeted specifically to erythroid tissue. Thus, there remains a need for improved vectors for gene delivery to these tissues.

SUMMARY OF THE INVENTION

The present invention provides an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein the cassette is located between the inverted terminal repeats, the selected DNA sequence is operably linked to the promoter, and the vector lacks any AAV structural genes. In preferred embodiments, the inverted terminal repeats comprises nucleotides 1 to 125 of SEQ ID NO:1. In other preferred embodiments, the promoter may be selected from the group consisting of CMV IE, LTR, SV40 IE, HSV *tk*, β-actin, b19p6, human globin α, human globin β and human globin γ promoter. In specific embodiments, the selected DNA sequence encodes a polypeptide. In particular embodiments,

the polypeptide may be selected from the group consisting of a gene encoding alpha-globin, beta-globin, gamma -globin, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, neomycin resistance, luciferase, adenine phosphoribosyl transferase (APRT), retinoblastoma, insulin, mast cell growth factor, p53, p21, p16 and adenosine deaminase.

In alternate embodiments, the selected DNA encodes an antisense RNA. More particularly, the antisense RNA is complementary to a segment of an oncogene. The oncogene may be selected from the group consisting of *myb*, *myc*, *ras*, *raf*, *erb*, *src*.

In another aspect, the present invention provides a B19 viral particle comprising an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein the cassette is located between the inverted terminal repeats, the selected DNA sequence is operably linked to the promoter, and the vector lacks any AAV structural genes.

In yet another embodiment, there is provided a helper virus construct comprising two adenovirus inverted terminal repeats, an AAV *rep* gene and a B19 VP2 gene, wherein the *rep* and *cap* genes are under the control of at least one promoter and are located between the inverted terminal repeats. In particular embodiments, the VP2 gene is under the control of the CMV IE promoter. In other embodiments, the *rep* gene is under the control of the B19 p5 promoter. In additional embodiments, it is contemplated that the helper virus further may comprise a B19 VP1 gene.

Also contemplated by the present invention is a method for packaging an AAV expression vector comprising the steps of providing an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein the cassette is located between the inverted terminal repeats, wherein the selected DNA sequence is operably linked to the promoter, and the vector lacks any AAV structural genes; providing a helper virus

construct comprising two adenovirus inverted terminal repeats, an AAV *rep* gene and a B19 VP2 gene, wherein the *rep* and *cap* genes are under the control of at least one promoter and are located between the inverted terminal repeats; contacting the expression vector and the helper virus construct with the host cell under conditions permitting the uptake of the expression vector and the helper virus construct by the cell; infecting the transfected host cell with adenovirus; and harvesting B19 particles from the host cell. In additional embodiments, it is contemplated that the helper virus further may comprise a B19 VP1 gene.

In particular embodiments, the multiplicity of infection of the adenovirus is about 10 pfu. Of course, this is an exemplary number, it is understood that values greater or less than this value also will be useful. Thus MOIs of 5 pfu, 6 pfu, 7 pfu, 8 pfu, 9 pfu, 10 pfu, 11 pfu, 12 pfu, 13 pfu, 14 pfu, 15 pfu, 16 pfu, 17 pfu, 18 pfu, 19 pfu, 20 pfu, 25 pfu, 30 pfu per cell or more may also be employed. In particular embodiments, the host cells are infected about 8 hours after transfer of the expression vector and the helper virus construct. The contacting conditions may comprise calcium phosphate precipitation, electroporation, microprojectile bombardment or lipofection. In other embodiments, the harvesting comprises host cell disruption, virus isolation and heat inactivation. In more particular aspects, the host cell disruption comprises freeze-thawing. In particular embodiments, the harvesting is performed between about 65 and about 72 hours after infection of the adenovirus. In preferred embodiments, the virus isolation may comprise a technique selected from the group consisting of centrifugation, filtration and ion exchange chromatography.

The invention also provides a method for expressing a selected DNA sequence in a host cell comprising the steps of providing a B19 viral particle comprising an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein the cassette is located between the inverted terminal repeats, wherein the selected DNA sequence is operably linked to the promoter, and the vector lacks any AAV structural genes; contacting the viral particle with the host cell under conditions permitting infection of the host cell; and culturing the host cell under conditions permitting the transcription of the DNA sequence. In particular embodiments, the DNA sequence encodes a polypeptide, in other

embodiments the DNA sequence encodes an antisense mRNA. In preferred embodiments, the host cell is an erythroid cell. In more preferred embodiments, the erythroid cell is a human erythroid cell.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 - Diagrammatic representation of construction of recombinant parvovirus B19-*lacZ*. pCMVp-*lacZ* contains a CMV promoter linked to the *lacZ* gene, flanked by AAV ITR's (SEQ ID NO:1). pSP-42 contains AAV *rep* and B19 *cap* genes under the endogenous promoter (SEQ ID NO:2) and the CMV promoter, respectively, and flanked by Ad ITR's. 293 cells are transfected with both plasmids, thereby producing a B19-like particle containing an AAV-like genome, lacking all coding regions other than CMVp-*lacZ*.

FIG. 2 - Electron microscopy of chimeric viral particles. The recombinant virions were purified on CsCl gradients as described in Example 1. Samples were negatively stained with 3% phosphotungstic acid (pH 6.5), and the particles were visualized using at a magnification of x80,000 using a Philips 400 electron microscope (bar = 80 nm).

FIG. 3A, FIG. 3B, FIG. 3C, FIG. 3D, FIG. 3E and FIG. 3F - Expression of the transduced *lacZ* gene mediated by the recombinant AAV- and B19-*lacZ* vectors in human 293

and Epo-differentiated MB-02 cells. Approximately equivalent numbers of 293 (FIG. 3A, FIG. 3B and FIG. 3C) and Epo-differentiated MB-02 cells (FIG. 3D, FIG. 3E and FIG. 3F) were either mock-infected or infected with 200 particle/cell each of AAV-*lacZ* (FIG. 3B and FIG. 3E) and B19-*lacZ* (FIG. 3C and FIG. 3F) recombinant vectors under identical conditions. Forty-eight hrs post-infection, the cells were fixed and stained for the analysis of expression of the *lacZ* gene as previously described (Ponnazhagan, 1996).

FIG. 4 - FACS analysis of the *lacZ* gene expression in glycophorin-positive primary human low-density bone marrow (LDBM) cells transduced with recombinant AAV- and B19-*lacZ* vectors. Glycophorin-positive cells from human LDBM were either mock-infected or infected with either $1x10^5$ particles/cell of AAV-*lacZ* vector, or $2x10^2$ particles/cell of B19-*lacZ* vector under identical conditions. Forty-eight hrs post-infection, cells were harvested and processed for analysis of the *lacZ* gene expression using FITC-conjugated X-Gal substrate using a Becton-Dickinson FACScanner as described in Example 1.

FIG. 5 - FACS analysis of the *lacZ* gene expression in erythroid and non-erythroid populations of primary human bone marrow-derived $CD34^+$ cells transduced with AAV- and B19-*lacZ* recombinant vectors. $CD34^+$ cells isolated from human LDBM were either mock-infected or infected with either $1x10^5$ particles/cell of the AAV-*lacZ* vector or $2x10^2$ particles/cell of the B19-*lacZ* vector under identical conditions. Forty-eight hrs post-infection, cells were harvested and processed for analysis of the *lacZ* gene expression in erythroid (stippled bars) and non-erythroid (shaded bars) using FITC-conjugated X-Gal and PE-conjugated glycophorin substrates, respectively, as described in Example 1.

FIG. 6 - FACS analysis of the *lacZ* gene expression in erythroid- and myeloid-differentiated $CD34^+$ primary human bone marrow cells transduced with AAV- and B19-*lacZ* recombinant vectors. Primary human bone marrow-derived $CD34^+$ cells were allowed to undergo differentiation into erythroid or myeloid lineages with the use of respective cytokine combinations for 10 days *in vitro* as described in Example 1. Following differentiation, cells were either mock-infected or infected with either $1x10^5$ particles/cell of the AAV-*lacZ* vector

or $2x10^2$ particles/cell of the B19-*lacZ* vector under identical conditions. Forty-eight hrs post-infection, cells were harvested and processed for analysis of the *lacZ* gene expression in erythroid (stippled bars) and myeloid (closed bars) populations using the FITC-conjugated X-Gal substrate as described in the legend to FIG. 4.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Viral vectors are widely utilized for a variety of gene transfer endeavors. For example, retroviral vectors have been used for a number of years to transform cell lines *in vitro* for the purpose of expressing exogenous polypeptides. More recently, with advancements in genetic therapies, various other vectors including adenoviruses and herpesviruses, along with retroviruses, have been utilized to transfer therapeutic genes into the cells of patients.

A variety of problems remain with respect to the use of viral vectors. First, many vectors are toxic to cells, and some may even be oncogenic. Second, expression of even non-toxic viral gene products may lead to the development of an immune response in the patient, thereby limiting the efficacy of the treatment and possibly preventing repeat administrations. Third, many viruses have limited host ranges and some may only replicate in dividing cells. Fourth, diminished transgene expression over time may further limit the effectiveness of the vector. And fifth, it may be difficult to generate viral vectors with sufficient titers to make treatments possible. All of these limitations have, in one case or another, hampered efforts by researchers to implement gene therapy. The present invention seeks to address some of these shortcomings by providing a new vector system specifically designed for the transformation of erythroid cells.

A. The Present Invention

In the present study, the inventors exploited the unique features of the AAV and B19 viruses to create a chimeric, parvovirus-based recombinant vector system. This system does not suffer from toxicity, oncogenicity and immunogenicity concerns, as do many other viral vectors. In addition, high titers of this vector may be generated, facilitating *in vivo* therapy. Further, this system is designed to specifically target primitive progenitor and differentiated

cells of erythroid lineage, and can achieve stable integration and expression of transduced genes.

Production of the recombinant vector is achieved by creating a helper plasmids of the *rep* gene sequences of AAV and the *cap* gene sequences of B19. The AAV-*rep* gene, in one example, is expressed using its own promoter, designated p5, based on the map unit determination, and the VP2 capsid gene of B19 is expressed under the control of a strong promoter such as the cytomegalovirus immediate early (CMV IE) gene promoter. This helper plasmid is cotransfected, along with a recombinant AAV plasmid containing either the bacterial β-galactosidase gene or human β-globin gene sequences, and a thymidine kinase (TK) promoter-driven bacterial neomycin resistance gene, cloned between a pair of AAV ITRs, into 293 cells by calcium phosphate precipitation protocol, which cells subsequently are infected with adenovirus. Harvesting, purification and heat inactivation of the recombinant virus are then performed.

The construction of these vectors, in various different embodiments, along with their potential uses, are described in greater detail below.

B. Adeno-Associated Virus

AAV utilizes a linear, single-stranded DNA of about 4700 base pairs. Inverted terminal repeats flank the genome. Two genes are present within the genome, giving rise to a number of distinct gene products. The first, the *cap* gene, produces three different virion proteins (VP), designated VP-1, VP-2 and VP-3. The second, the *rep* gene, encodes four non-structural proteins (NS). One or more of these *rep* gene products is responsible for transactivating AAV transcription. The sequence of AAV is provided by Srivastava *et al.*, (1983) and in U.S. Patent 5,252,479 (entire text of which is specifically incorporated herein by reference).

The three promoters in AAV are designated by their location, in map units, in the genome. These are, from left to right, p5, p19 and p40. Transcription gives rise to six transcripts, two initiated at each of three promoters, with one of each pair being spliced. The splice site, derived from map units 42-46, is the same for each transcript. The four non-

structural proteins apparently are derived from the longer of the transcripts. and three virion proteins all arise from the smallest transcript.

AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV *rep* proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

The terminal repeats of the AAV vector of the present invention can be obtained by restriction endonuclease digestion of AAV or a plasmid such as p201, which contains a modified AAV genome (Samulski *et al.* (1987). Alternatively, the terminal repeats may be obtained by other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis of the terminal repeats based upon the published sequence of AAV. The ordinarily skilled artisan can determine, by well-known methods such as deletion analysis, the minimum sequence or part of the AAV ITRs which is required to allow function, *i.e.*, stable and site-specific integration. The ordinarily skilled artisan also can determine which minor modifications of the sequence can be tolerated while maintaining the ability of the terminal repeats to direct stable, site-specific integration.

C. B19 Virus

While AAV causes no known disease, B19 is known to be the etiologic agent of a variety of serious clinical disorders in humans. For example, B19 is the causative agent of transient aplastic crises associated with various hemolytic anemias, erythema infectiosum or the "fifth disease," post-infection polyarthralgia and thrombocytopenia in adults, and some cases of chronic bone marrow failure and hydrops fetalis.

Structurally, the B19 virus is very similar to AAV, having *rep* and *cap* genes flanked by ITRs. One notable difference between the two is the transcriptional pattern, which results in nine different transcripts, all initiated at a single start site at the left end of the genome.

Another difference is the 3' ITR, which for most parvoviruses is about 115 bases, but in the case of B19, is more than 300 bases in length.

In particular, the present invention utilizes the *cap* gene sequences of the B19 virus. This gene encodes the structural components of the B19 virion and provides the host cell specificity of the virus. Relevant sequences from B19 may be obtained from B19 DNA or cloned B19 DNA. See, for example, Cotmore *et al.* (1984); Shade *et al.* (1986). Standard techniques for the construction of such B19 vectors are well-known to those of ordinary skill in the art and can be found in references such as Sambrook *et al.* (1989), or any of the myriad of laboratory manuals on recombinant DNA technology that are widely available. A variety of strategies are available for restricting and ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and can be readily determined by the skilled artisan.

The present invention provides a unique chimeric vector system in which heterologous DNA sequences are cloned within the inverted terminal repeats (ITR) of AAV, and subsequently packaged inside the capsid structure of B19. Production of such a recombinant chimeric vector is achieved by creating a helper plasmid consisting the *rep* gene sequences of AAV and the *cap* gene sequences of B19. The AAV-*rep* was expressed using its own promoter, termed p5, based on the map unit determination and the VP2 capsid gene of B19 was expressed under the control of cytomegalovirus early gene promoter. Thus, the B19 for the purposes of the present invention, needs only to have a *cap* gene that is capable of facilitate packaging. Thus, the cap gene may be a VP2 gene alone or in combination with the B19 VP1 gene. These *cap* genes may be wild-type or a recombinant genes. Additional disclosure regarding B19 parvovirus capsids and genes thereof may be found in U.S. Patent 5,508,186 (specifically incorporated herein by reference) which describes recombinant baculoviruses encoding parvovirus structural proteins, method of infecting host cells therewith, and methods of packaging and delivering genetic information utilizing the noninfectious capsids.

D. Adenovirus

Adenovirus is a linear, double-stranded DNA virus with a genome of about 36 kB. Adenovirus can infect a wide range of host cells in a non-integrative fashion. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans. Both ends of the viral genome contain 100-200 base pair inverted terminal repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

Recently, Racher *et al.*, (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, adenovirus is relatively easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{11} plaque-forming units per ml, and they are highly infectious. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

E. AAV and Adenovirus Hybrid Vectors

In accordance with the present invention, two vectors are provided that, when introduced into the same cell, produce an apathogenic, recombinant, chimeric parvovirus capable of targeting cells of erythroid origin. The first vector is derived primarily from AAV and carries a recombinant DNA construct comprising a heterologous gene to be delivered to a target cell. The second vector is derived from adenovirus, containing ITRs from this virus, but contains AAV and B19 sequences as well. This second vector serves to provide structural and replicative functions that facilitate the packaging of the first vector.

The AAV-derived vectors comprise a first and second AAV ITR, which flank at least a first promoter operably linked to a heterologous gene. The terminal repeats can comprise all or an active portion of the ITRs of AAV. By active, it is meant that sufficient portions of the ITR exists to permit replication and packaging of the vector. Further, the ITRs mediate stable integration of the DNA sequence into a specific site in a particular chromosome, specifically, on human chromosome 19. The entire DNA sequence, including the ITRs, the promoter, and

the heterologous gene, is integrated into the genome. Therefore, in preferred embodiments, the ITRs or portions thereof also permit integration.

The hybrid Ad-AAV-B19-Ad vectors of the present invention comprise a first and a second adenovirus ITR, which flank an AAV *rep* gene and a B19 *cap* gene. Preferably, promoters are provided for each of the *rep* and *cap* genes. Again, the terminal repeats may comprise all or an active portion of the ITRs of adenovirus, with active defined, in this instance, as permitting the replication of the vector. Packaging of the vector into a viral capsid is not necessary as the DNA can be transfected into cells along with the AAV-derived vector, although in some embodiments, it may be desired that this hybrid vector be packaged in an adenoviral particle. In this situation, an active adenoviral packaging signal is necessary. This signal is located at approximately 290-390 bases pairs from the left end of the genome.

F. Propagation of Vectors and Transformation of Host Cells

The following is an exemplary description of the propagation of vectors of the present invention, of course the conditions described are only exemplary and in light of the present disclosure it will be possible for one of ordinary skill in the art to modify these propagation conditions according to particular needs. Approximately 10 μg each of the recombinant AAV plasmid and the AAV- or B19-helper plasmids are co-transfected per confluent 100 mm dish of 293 cells. Transfected cells are also infected with 10 plaque-forming units (pfu) per cell of human adenovirus type 2 (Ad2). Crude cell lysates are prepared 65-72 hrs post-infection by three cycles of freezing and thawing followed by heat-inactivation of Ad2 at 56°C for 30 min. Clarified supernatants are digested with DNase I (100 U/ml) for 1 hr at 37°C and adjusted to a density of 1.40 g/ml by addition of CsCl and centrifuged at 35,000 rpm for 40 hrs at 20°C. Equilibrium density gradients are fractionated by collecting drops through a puncture in the bottom of the centrifuge tube. The densities of all fractions are determined from refractive index measurements. All fractions are dialyzed against 1xSSC (0.15 M NaCl, 0.015 M sodium citrate) and analyzed for viral DNA by slot blot analysis. Viral titers of rAAV generally ranges from 10^{11} to 10^{13} particles/ml, and for rB19 from 10^{8} to 10^{9} particles/ml.

Virions can be produced by cotransfer of the helper plasmid and the AAV plasmid, followed by infection with a helper virus such as adenovirus, herpes virus or vaccinia virus. Transfer may be accomplished any standard gene transfer mechanism: calcium phosphate precipitation, lipofection, electroporation, microprojectile bombardment or other suitable means. Following transfer, host cells are infected with a helper virus, virions are isolated and the helper virus is inactivated (*e.g.*, heated at 56°C for one hour). The resulting helper free stocks of virions are used to infect appropriate target cells. Mature virions may further be isolated by standard methods, *e.g.*, cesium chloride centrifugation, and to inactivate any contaminating adenovirus.

Bone Marrow Cells are an example of host cells that may be transformed conditions for transformation of host cells. Equivalent numbers of primary human low-density bone marrow (LDBM) mononuclear cells are either mock-infected or infected with 1,000 - 10,000 particles/cell of rAAV at 37°C for 1 hr, washed with sterile phosphate-buffered saline (PBS), and incubated in IMDM containing 20% FBS and antibiotics at 37°C in a 5% CO_2 incubator. The LDBM and differentiated and undifferentiated CD34+ cells are either mock-infected or infected with the recombinant B19-lacZ or recombinant AAV-lacZ vectors at a particle to cell ratio of 200:1 and 100,000:1, respectively, at 37°C for 1 hr following which the cells are grown in the presence of cytokines (interleukin-3 (IL-3), interleukin-6 (IL-6) and stem cell factor (SCF)) for a period of 48 hrs. Analysis of expression of the transduced lacZ gene is performed using the FITC-conjugated substrate for beta-Gal and the PE-conjugated substrates for CD34 and CD33 antigens or glycophorin. Briefly, cells are incubated with 300 μM chloroquine for 30 min. at 30°C following which chloroquine is removed by centrifugation and cells are incubated further with 33 μM Imagreen C12FDG β-gal substrate for 30 min. at 37°C. Following centrifugation, the cells are resuspended in fresh culture medium and analyzed using a Beckton-Dickinson FACScanner.

In another embodiment, the present invention contemplates the use of helper vectors packaged in viral particles. In particular, the Ad-AAV-B19-Ad vectors can be engineered to contain sufficient *cis*-acting signals for their packaging in adenoviral capsids. The requirements for adenoviral packaging are well-defined by the field and largely reside in the adenoviral left

end ITR. The only other major concerns relate to the compatibility of inserted sequences and the overall size of the vector, which is up to about 40 kB. This system would require provision of adenoviral replicative and packaging functions *in trans*, either by a cell line or by a helper virus. Essential functions from adenovirus, which would need to be provided, include E1A, E1B, E2A, E2B, E4 and L1-L5. 293 cells are well known in the art and can provide E1A and E1B functions.

Function of the hybrid vectors of the present invention, *i.e.*, the ability to mediate transfer and expression of the heterologous gene in hematopoietic stem or progenitor cells, can be evaluated by monitoring the expression of the heterologous gene in transduced cells. Obviously, the assay for expression depends upon the nature of the heterologous gene. Expression can be monitored by a variety of methods including immunological, histochemical or activity assays. For example, Northern analysis can be used to assess transcription using appropriate DNA or RNA probes. If antibodies to the polypeptide encoded by the heterologous gene are available, Western blot analysis, immunohistochemistry or other immunological techniques can be used to assess the production of the polypeptide. Appropriate biochemical assays can also be used if the heterologous gene is an enzyme. For example, if the heterologous gene encodes antibiotic resistance, a determination of the resistance of infected cells to the antibiotic can be used to evaluate expression of the antibiotic resistance gene.

Site-specific integration can be assessed, for example, by Southern blot analysis. DNA is isolated from cells transduced by the vectors of the present invention, digested with a variety of restriction enzymes, and analyzed on Southern blots with an AAV-specific probe. A single band of hybridization evidences site-specific integration. Other methods known to the skilled artisan, such as polymerase chain reaction (PCR™) analysis of chromosomal DNA can be used to assess stable integration.

G. Promoters

In describing both the hybrid vector construct, which contains *cap* and *rep* genes, and the AAV-derived plasmid, which contains the transgene of interest, it should be noted that genetic elements will be required to drive the transcription of genes therein. Thus, the nucleic

acid encoding a gene product is placed under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "under transcriptional control" or "operably linked" mean that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of direction the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of genes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product. Tables 2 and 3 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of the gene of interest. This list is not intended to be exhaustive of all the possible elements involved in the promotion of gene expression but, merely, to be exemplary thereof.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. Thus, in certain situations, it will be desirable to further include an enhancer in the regulatory cassette.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or

more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct (Table 1 and Table 2). Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

ENHANCER/PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β-Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DRα
β-Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase *I*
Metallothionein
Collagenase
Albumin Gene

TABLE 1 - Continued

ENHANCER/PROMOTER
α-Fetoprotein
τ-Globin
β-Globin
e-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 2

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
ß-Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H_2O_2
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone
Insulin E Box	Glucose

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

(i) Transgene Constructs

Transgene expression will be driven by a selected promoter. The promoter selection will depend on the polypeptide to be expressed, the target tissue and the purpose for expression. For example, if the protein is simply to be produced *in vitro* and purified, a high level promoter will be utilized. If the protein is toxic to the cells, it may be desirable to regulate the expression of the protein such that cell proliferation is maximized prior to polypeptide expression. If the protein's processing or secretion is dependent upon a particular stage in the host cell's cycle, it may be desirable to employ a promoter that is regulated in an appropriate, cell cycle dependent fashion.

For example, the B19 p6 promoter provides for expression specific to erythroid progenitor cells. The nucleotide sequence of B19 from nucleotide number 200 to nucleotide number 424, as numbered by Shade *et al.* (1986), contains the p6 promoter. A consensus promoter-like sequence TATATATA is located at nucleotide 320 in B19 and, thus, transcription is likely to originate about 30 nucleotides downstream. It is known that B19 fragments containing these sequences direct expression that is specific for erythroid progenitor cells, and that deletion of B19 coding sequences downstream from the promoter prevents replication of B19.

One of ordinary skill in the art can determine the minimum sequence and modifications of the p6 promoter which provide cell-specific, non-cytotoxic expression. This can be determined by infecting erythroid and non-erythroid cells with vectors containing the p6 promoter and assessing expression of the heterologous gene. The promoter sequence can be derived by restriction endonuclease digestion of B19 or a cloned B19 plasmid such as pYT103 and pYT107 (Cotmore *et al.* (1984)) or by any other methods known to the skilled artisan, including but not limited to chemical or enzymatic synthesis based upon the published sequence of B19.

Other cell-specific promoters can be obtained by analogous methods, and the specificity of these promoters is determined by assessing expression in the appropriate cell type.

(ii) Rep and Cap Gene Constructs

The vectors of the present invention include a "helper" vector that supplies the replication (*rep*) function of AAV and the capsid (*cap*) function of B19. Each of these genes may be contained in a single expression cassette. The exemplified construct utilizes a CMV IE promoter operably linked to the *cap* gene, and a the p5 promoter of AAV operably linked to the *rep* gene. Where multigene constructs are utilized, internal ribosome entry sites (IRES) may increase the level and fidelity of expression of the downstream gene (discussed below).

Generally, any high level expression promoter will suffice to drive these genes. However, certain advantages may accrue through the use of the homologous (normal) promoter for these genes and/or viruses. For example, position effects in the 5′ untranslated portion of a gene may be difficult to duplicate in synthetic constructs and may be responsible for dramatic variations in expression levels. Similarly, given the dependence of the present vectors on superinfecting helper viruses, it may prove advantageous to utilize the homologous promoter. As mentioned above, the B19 p6 promoter is very useful in the context of the present invention, and is a homologous promoter with respect to the B19 *cap* gene. Another homologous promoters include the AAV p5 promoter for the AAV *rep* gene.

(iii) Selectable Markers

In certain embodiments of the invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the vector and, hence, the gene of interest. Usually, the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase or chloramphenicol acetyltransferase (CAT) may be employed. Further examples of selectable markers are well known to one of skill in the art.

(iv) IRES

In certain embodiments of the present invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′–methylated Cap-dependent translation

and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picanovirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

H. Transgenes

Virtually any transgene may be utilized in the vectors described herein. In a preferred embodiment, the heterologous gene encodes a biologically functional protein, *i.e.*, a polypeptide or protein which affects the cellular mechanism of a cell in which the biologically functional protein is expressed. For example, the biologically functional protein can be a protein which is essential for normal growth of the cell or for maintaining the health of a mammal. The biologically functional protein can also be a protein which improves the health of a mammal by either supplying a missing protein, by providing increased quantities of a protein which is underproduced in the mammal or by providing a protein which inhibits or counteracts an undesired molecule which may be present in the mammal. The biologically functional protein can also be a protein which is a useful protein for investigative studies for developing new gene therapies or for studying cellular mechanisms.

(i) Secreted Proteins

The cDNA's encoding a number of useful human proteins are available for insertion into vectors of the present invention. Examples would include soluble CD4, Factor VIII, Factor IX,

von Willebrand Factor, TPA, urokinase, hirudin, interferons α and β, TNF, GM-CSF, antibodies, albumin, transferin and nerve growth factors. Cytokines such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 also are contemplated.

Peptide hormones are grouped into three classes with specific examples given for each. These classes are defined by the complexity of their post-translational processing. Class I is represented by Growth Hormone, Prolactin and Parathyroid hormone. A more extensive list of human peptides that are included in Class I is given in Table 3. These require relatively limited proteolytic processing followed by storage and stimulated release from secretory granules. Class II is represented by Insulin and Glucagon. A more extensive list of human peptide hormones that are included in Class II are given in Table 4. Further proteolytic processing is required, with both endoproteases and carboxypeptidases processing of larger precursor molecules occurring in the secretory granules. Class III is represented by Amylin, Glucagon-like Peptide I and Calcitonin. Again, a more extensive list of Class III human peptide hormones is given in Table 5. In addition to the proteolytic processing found in the Class II peptides, amidation of the C-terminus is required for proper biological function. Examples of engineering expression of all three of these classes of peptide hormones in a neuroendocrine cell are found in this patent.

TABLE 3

Class I Human Peptide Hormones

Growth Hormone	Follicle-stimulating Hormone
Prolactin	Chorionic Gonadotropin
Placental Lactogen	Thyroid-stimulating Hormone
Luteinizing Hormone	Leptin

TABLE 4

Human Peptide Hormones Processed by Endoproteases from Larger Precursors

Adrenocorticotropin (ACTH)	Gastric Inhibitory Peptide (GIP)
Angiotensin I and II	Glucagon
β-endorphin	Insulin
Cholecystokinin	Lipotropins
Endothelin I	Neurophysins
Galanin	Somatostatin
β-Melanocyte Stimulating Hormone (β-MSH)	

TABLE 5

Amidated Human Peptide Hormones

Calcium Metabolism Peptides:

- Calcitonin
- Calcitonin Gene related Peptide (CGRP)
- β-Calcitonin Gene Related Peptide
- Hypercalcemia of Malignancy Factor (1-40) (PTH-rP)
- Parathyroid Hormone-related protein (107-139) (PTH-rP)
- Parathyroid Hormone-related protein (107-111) (PTH-rP)

Gastrointestinal Peptides:

- Cholecystokinin (27-33) (CCK)
- Galanin Message Associated Peptide, Preprogalanin (65-105)
- Gastrin I
- Gastrin Releasing Peptide
- Glucagon-like Peptide (GLP-1)
- Pancreastatin
- Pancreatic Peptide
- Peptide YY

TABLE 5 - Continued

Gastrointestinal Peptides - continued:

PHM

Secretin

Vasoactive Intestinal Peptide (VIP)

Pituitary Peptides:

Oxytocin

Vasopressin (AVP)

Vasotocin

Enkephalins:

Enkephalinamide

Metorphinamide (Adrenorphin)

Alpha Melanocyte Stimulating Hormone (alpha-MSH)

Atrial Natriuretic Factor (5-28) (ANF)

Amylin

Amyloid P Component (SAP-1)

Corticotropin Releasing Hormone (CRH)

Growth Hormone Releasing Factor (GHRH)

Luteinizing Hormone-Releasing Hormone (LHRH)

Neuropeptide Y

Substance K (Neurokinin A)

Substance P

Thyrotropin Releasing Hormone (TRH)

(ii) Non-Secreted Proteins

Two general classes of non-secreted proteins can be defined. The first are proteins that, once expressed in cells, stay associated with the cells in a variety of destinations. These destinations include the cytoplasm, nucleus, mitochondria, endoplasmic reticulum, golgi,

membrane of secretory granules and plasma membrane. Non-secreted proteins are both soluble and membrane associated. The second class of proteins are ones that are normally associated with the cell, but have been modified such that they are now secreted by the cell. Modifications would include site-directed mutagenesis or expression of truncations of engineered proteins resulting in their secretion as well as creating novel fusion proteins that result in secretion of a normally non-secreted protein.

The cDNA's encoding a number of therapeutically useful human proteins are available. These include cell surface receptors, transporters and channels such as GLUT2, CFTR, leptin receptor, sulfonylurea receptor, β-cell inward rectifying channels, *etc.* Other proteins include protein processing enzymes such as PC2 and PC3, and PAM, transcription factors such as IPF1, and metabolic enzymes such as adenosine deaminase, phenylalanine hydroxylase, glucocerebrosidase.

Engineering mutated, truncated or fusion proteins also is contemplated. Examples of each type of engineering resulting in secretion of a protein are given (Ferber *et al.*, 1991; Mains *et al.*, 1995). Reviews on the use of such proteins for studying the regulated secretion pathway are also cited (Burgess and Kelly, 1987; Chavez *et al.*, 1994).

In addition to the proteins discussed above, there are proteins that fall into various classes that would be suitable for producing transgenic constructs in the present invention. Such classes include tumor suppressors, inducers of apoptosis, enzymes, cytokines, enzymes and toxins, the following section describes examples of each of these classes. This list is not intended to be exhaustive, rather it is intended to provide examples of the types of proteins that may be encoded by transgenes using the vectors of the present invention.

Tumor Suppressors. p53 currently is recognized as a tumor suppressor gene. High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most

frequently-mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as SV40 large-T antigen and adenoviral E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus, p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild-type p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene.

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53, in as much as mutations in p53 are known to abrogate the tumor suppressor capability of wild-type p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey *et al.*, 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahasi *et al.*, 1992). p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 does not affect the growth of normal or

non-malignant cells with endogenous p53. Thus, such constructs might be taken up by normal cells without adverse effects. It is thus proposed that the treatment of p53-associated cancers with wild-type p53 will reduce the number of malignant cells or their growth rate.

The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G_1. The activity of this enzyme may be to phosphorylate Rb at late G_1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit $p16^{INK4}$. The $p16^{INK4}$ has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the $p16^{INK4}$ protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

$p16^{INK4}$ belongs to a newly described class of CDK-inhibitory proteins that also includes $p15^{INK4B}$, $p21^{WAF1}$, and $p27^{KIP1}$. The $p16^{INK4}$ gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the $p16^{INK4}$ gene are frequent in human tumor cell lines. This evidence suggests that the $p16^{INK4}$ gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the $p16^{INK4}$ gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). However, it was later shown that while the p16 gene was intact in many primary tumors, there were other mechanisms that prevented p16 protein expression in a large percentage of some tumor types. p16 promoter hypermethylation is one of these mechanisms (Merlo *et al.*, 1995; Herman, 1995; Gonzalez-Zulueta, 1995). Restoration of wild-type $p16^{INK4}$ function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995). Delivery of p16 with adenovirus vectors inhibits production of some human cancer lines and reduces the growth of human tumor xenografts

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.

Cell adhesion molecules, or CAM's are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAM's maybe involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matsura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells *in vivo*. C-CAM now has been shown to suppress tumor growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include p21, p15, BRCA1, BRCA2, IRF-1, PTEN, RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, FCC and MCC.

Inducers of Apoptosis. Inducers of apoptosis, such as Bax, Bak, Bcl-X_S, Bad, Bim, Bik, Bid, Harakiri, Ad E1B, Bad and ICE-CED3 proteases, similarly could find use according to the present invention.

Enzymes. Various enzyme genes are of interest according to the present invention. Such enzymes include cytosine deaminase, adenosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridyltransferase, phenylalanine hydroxylase, glucocerbrosidase, sphingomyelinase, α-L-iduronidase, glucose-6-phosphate dehydrogenase,

HSV thymidine kinase and human thymidine kinase and extracellular proteins such as collagenase and matrix metalloprotease.

Cytokines. Another class of genes that is contemplated to be inserted into the adenoviral vectors of the present invention include interleukins and cytokines. Interleukin 1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, β-interferon, α-interferon, γ-interferon, angiostatin, thrombospondin, endostatin, METH-1, METH-2, GM-CSF, G-CSF, M-CSF and tumor necrosis factor.

Toxins. Various toxins are also contemplated to be useful as part of the expression vectors of the present invention. these toxins include bacterial toxins such as ricin A-chain (Burbage, 1997), diphtheria toxin A (Massuda *et al.*, 1997; Lidor, 1997), pertussis toxin A subunit, *E. coli* enterotoxin toxin A subunit, cholera toxin A subunit and pseudomonas toxin c-terminal. Recently, it was demonstrated that transfection of a plasmid containing the fusion protein regulatable diphtheria toxin A chain gene was cytotoxic for cancer cells. Thus, gene transfer of regulated toxin genes might also be applied to the treatment of cancers (Massuda *et al.*, 1997).

(iii) Antisense Constructs

In some cases, one may wish to block the function or expression of a particular polypeptide. Antisense constructs are one way of addressing this situation. Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see below) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

(iv) Ribozyme Constructs

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech. 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent No. 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

I. Methods for Production of Polypeptides *In Vitro*

In one embodiment, the present invention contemplates the use of chimeric parvovirus vectors to transform cells for the production of mammalian cell cultures. In order for the cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells are maintained with the correct ratio of oxygen and carbon dioxide and nutrients, but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

The construct encoding the protein of interest may be transferred by the viral vector, as described above, into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. Examples of useful mammalian cell lines are those that express the appropriate receptor for B19 virus. These include bone marrow cells, peripheral blood cells, endothelial cells and myocardial cells (Rouger *et al.*, 1987; van dem Borne *et al.*, 1986)

Bone marrow cells are isolated and enriched for hematopoietic stem cells (HSC), *e.g.*, by fluorescence activated cell sorting as described in Srivastava *et al.* (1988). HSC are capable of self-renewal as well as initiating long-term hematopoiesis and differentiation into multiple hematopoietic lineages *in vitro*. HSC are transfected with the vector of the present invention or infected with varying concentrations of virions containing a subject hybrid vector and then assessed for the expression of the heterologous gene. The assay for expression depends upon the nature of the heterologous gene. Expression can be monitored by a variety of methods including immunological, histochemical or activity assays. For example, Northern analysis can be used to assess transcription using appropriate DNA or RNA probes. If antibodies to the polypeptide encoded by the heterologous gene are available, Western blot analysis, immunohistochemistry or other immunological techniques can be used to assess the production of the polypeptide. Appropriate biochemical assays can also be used if the heterologous gene is an enzyme. For example, if the heterologous gene encodes antibiotic resistance, a determination of the resistance of infected cells to the antibiotic can be used to evaluate expression of the antibiotic resistance gene.

An important consideration is the appropriate modification needed for a particular polypeptide. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the protein expressed.

A number of selection systems may be used including, but not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in *tk-*, *hgprt-* or *aprt-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

Large scale suspension culture of mammalian cells in stirred tanks is a common method for production of recombinant proteins. Two suspension culture reactor designs are in wide use - the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon. Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or

indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

J. Methods of Purifying Proteins

In another aspect of the invention, it will be desirable to purify polypeptides produced by culture of cells infected with the chimeric parvoviruses described herein. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur. Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major

component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

of homology between the two sequences suggest that human GDF-9 plays an important role during embryonic development and/or in the adult ovary.

EXAMPLE 5

NUCLEIC ACID DETECTION OF EXPRESSION OF GDF-9 IN OOCYTES

In order to localize the expression of GDF-9 in the ovary, *in situ* hybridization to mouse ovary sections was carried out using an antisense GDF-9 RNA probe. FIGURE 7 shows *in situ* hybridization to adult ovary sections using a GDF-9 RNA probe. [^{35}S]-labeled anti-sense (FIGURE 7a and 7c) or sense (FIGURE 7b and 7d) GDF-9 RNA probes were hybridized to adjacent paraffin-embedded sections of ovarians fixed in 4% paraformaldehyde. Sections were dipped in photographic emulsion, exposed, developed, and then stained with hematoxylin and eosin. Two representative fields are shown.

As shown in FIGURES 7a and 7c, GDF-9 mRNA was detected primarily in oocytes in adult ovaries. Every oocyte (regardless of the stage of follicular development) examined showed GDF-9 expression, and no expression was detected in any other cell types. No hybridization was seen using a control GDF-9 sense RNA probe (FIGURE 7b and 7d). Hence, GDF-9 expression appears to be oocyte-specific in adult ovaries.

To determine the pattern of expression of GDF-9 mRNA during ovarian development, sections of neonatal ovaries were probed with a GDF-9 RNA probe. FIGURE 8 shows *in situ* hybridization to a postnatal day 4 ovary section using an antisense GDF-9 RNA probe. Sections were prepared as described for FIGURE 7. Following autoradiography and staining, the section was photographed under bright-field (FIGURE 8a) or dark-field (FIGURE 8b) illumination.

FIGURE 9 shows *in situ* hybridization to postnatal day 8 ovary sections using an antisense (FIGURE 9a) or sense (FIGURE 9b) GDF-9 RNA probe. Sections were prepared as described for FIGURE 7.

specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Conconavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

K. Methods of Therapy

The hybrid vectors of the present invention are useful for gene therapy. In particular, the vectors of the present invention can direct erythroid cell-specific expression of a desired gene, and thus are useful in the treatment of hemoglobinopathies. Such maladies include thalassemia, sickle-cell anemia, diabetes, and cancer. The heterologous gene, in this context, can be the normal counterpart of one that is abnormally produced or underproduced in the disease state, for example β-globin for the treatment of sickle-cell anemia, and α-globin, β-globin or γ-globin

in the treatment of thalassemia. The heterologous gene can encode antisense RNA as described hereinabove. For example, alpha -globin is produced in excess over β-globin in β-thalassemia. Accordingly, β-thalassemia can be treated in accordance with the present invention by gene therapy with a vector in which the heterologous gene encodes an antisense RNA. The antisense RNA is selected such that it binds to a target sequence of the α-globin mRNA to prevent translation of α-globin, or to a target sequence of the α-globin DNA such that binding prevents transcription of α-globin DNA. In the treatment of cancer the heterologous gene can be a gene associated with tumor suppression, such as retinoblastoma gene, p53, p16, p21 or the gene encoding tumor necrosis factor.

The use of the hybrid vectors of the present invention for the treatment of disease involves, in one embodiment, the transduction of hematopoeitic stems cells or progenitor cells with the claimed vectors. Transduction is accomplished, following preparation of mature virions containing the AAV vectors, by infection of HSC or progenitor cells therewith. Transduced cells may be located in patients or transduced *ex vivo* and introduced or reintroduced into patients, *e.g.*, by intravenous transfusion. Rosenberg, 1990).

In *ex vivo* embodiments, HSC or progenitor cells are provided by obtaining bone marrow cells from patients and optionally enriching the bone marrow cell population for HSC. HSC can be transduced by standard methods of transfection or infected with mature virions for about one to two hours at about 37°C. Stable integration of the viral genome is accomplished by incubation of HSC at about 37°C for about one week to about one month. The stable, site-specific integration and erythroid cell-specific expression is assessed as described above. After the transduced cells have been introduced into a patient, the presence of the heterologous gene product can be monitored or assessed by an appropriate assay for the gene product in the patient, for example in peripheral red blood cells or bone marrow of the patient when expression is erythroid cell-specific. As described above, the specific assay is dependent upon the nature of the heterologous gene product and can readily be determined by one skilled in the art.

In a particular embodiment, β-thalassemia represents a heterologous group of clinical syndromes that are inherited as mutated alleles of genes that encode the human β-globin chain.

These mutations affect all aspects of β-globin gene expression including transcription, splicing, polyadenylation, translation, and protein stability. The hallmark of β-thalassemia is the marked reduction or total absence of synthesis of normal adult hemoglobin (HbA; α_2 β_2). Despite significant advances in the understanding of basic underlying molecular mechanisms of β-thalassemia, treatment is limited to regular red blood cell transfusions and iron-chelation therapy. Treatment by bone marrow transplantation has also been attempted (Thomas *et al.* 1982), but an effective cure has not been found.

Accordingly, the vectors of the present invention are useful in the treatment of β-thalassemia. An AAV-B19 vector is constructed in which the heterologous gene is the normal human β-globin gene, with the resulting AAV-B19- β-globin vector allowing parvovirus-mediated transfer, site-specific integration and erythroid cell-specific expression of the normal human beta -globin gene in human hematopoietic cells.

Abnormal beta-globin expression in beta-thalassemia may result in the overabundance of alpha-globin mRNA relative to beta-globin mRNA. The present invention can not only provide a normal beta-globin gene, as described hereinabove, but can further be utilized to down-regulate the production of excess alpha-globin by providing a vector with an antisense RNA as the heterologous gene.

Hence, the present invention contemplates gene therapy for β-thalassemia comprising transduction of hematopoietic stem or progenitor cells with a hybrid vector encoding normal β-globin chains, or simultaneous transduction with a vector encoding a normal β-globin chain and a vector encoding an RNA antisense to alpha -globin mRNA or DNA. Alternately, a construction with more than one B19 p6 promoter, as described hereinabove, permits coincident expression of β-globin and antisense α-globin. Accordingly, transduction with a single vector effects both the provision of a normal β-globin gene and the down-regulation of excess α-chains. More specifically, bone marrow cells are transfected with the subject vectors, and transduced cells are introduced, by intravenous transfusion, into a patient. The stable integration of the vector can be assessed by PCR™ or Southern blot analysis and the expression of the heterologous gene can be evaluated by assaying for the heterologous gene product in the

patient's peripheral blood cells or bone marrow cells. As described previously, the particular assay depends upon the nature of the heterologous gene product.

Yet another aspect of the present invention provides a method for delivery of a pharmaceutical product, a protein or an antisense RNA in a mammal. Since the normal differentiation of these stem cells results in production of mature erythrocytes, the transduction of stem cells with the subject vector ultimately yields a population of circulating, enucleate vesicles containing the gene product. This method comprises transducing hematopoietic stem or progenitor cells with the hybrid vector of the present invention and introducing, by intravenous transfusion or injection, the transduced cells into a mammal. Transduction can be accomplished by transfecting cells with the hybrid vector by standard methods or infecting cells with mature AAV virions containing the hybrid vector at about 37°C. for about one to two hours. Stable integration of the recombinant viral genome is accomplished by incubating cells at about 37°C. for about one week to about one month. Transduced cells are recognized by assaying for expression of the heterologous gene, as described hereinabove. In this embodiment, the pharmaceutical product is encoded by the heterologous gene of the hybrid vector, and can be any pharmaceutical product capable of being expressed by the hybrid vector. Such products include alpha, beta and gamma -globin, insulin, GM-CSF, M-CSF, G-CSF, EPO, TNF, MGF, interleukins, the gene product of the retinoblastoma gene, p53 or adenosine deaminase. The coding sequences of the respective genes are known (Lee *et al.* (1985; GM-CSF); Broderick *et al.*, (1987; APRT); Tratschin *et al.* (1985; Neo^r); Huang *et al.* (1988; RB-1); Liebhaber *et al.* (1980; α-globin); Lawn *et al.* (1980; β-globin); Enver *et al.* (1989; γ-globin)) and thus can be easily provided as described hereinabove. Therefore, the present invention can provide production of constitutive levels of heterologous gene products inside membrane vesicles, specifically red blood cells, for *in situ* treatment of disease. Optionally, the hybrid vector can further comprise a sequence which encodes a signal peptide or other moiety which facilitates the secretion of the gene product from the erythroid cell. Such sequences are well-known to one of ordinary skill in the art (Michaelis *et al.* 1982) and can be inserted into the subject vectors between the promoter and coding region by methods described herein above. This method can be used to treat a variety of diseases and disorders and is not limited to the treatment of hemoglobinopathies, since the heterologous gene is constitutively expressed and

can be released from the red blood cell by virtue of a secretory sequence, or released when red blood cells are lysed in the liver and spleen.

L. Pharmaceutical Compositions and Routes of Administration

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions containing the viral vectors of the present invention in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when viral preparations are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such

compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The active compounds also may be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In

the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

For oral administration the polypeptides of the present invention may be incorporated with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an antiseptic wash containing sodium borate, glycerin and potassium bicarbonate. The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

M. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Materials And Methods

Cells, plasmids and viruses:

The human nasopharyngeal carcinoma cell line, KB, was provided by A.C. Antony (Indiana University School of Medicine, Indianapolis, IN) and the human embryonic kidney

cell line, 293, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cell lines were maintained as previously described (Ponnazhagan *et al.*, 1996). The human megakaryocytic leukemia cell line, MB-02, a kind gift of D.A. Morgan (Hahnemann Medical College, Philadelphia, PA) was maintained and differentiated with erythropoietin (Epo) as previously described before (Morgan *et al.*, 1991; Munshi *et al.*, 1993). Human bone marrow cells were obtained from healthy volunteer donors after obtaining informed consent approved by the Institutional Review Board for studies involving human subjects. The recombinant AAV-helper plasmid, pAAV/Ad, and the recombinant B19 plasmid, pYT-103, were provided by R.J. Samulski (University of North Carolina, Chapel Hill, NC) and P. Tattersall (Yale University School of Medicine, New Haven, CT), respectively. The details of construction of the recombinant AAV plasmid containing the bacterial b-galactosidase (lacZ) gene under the control of the cytomegalovirus immediate-early promoter (pCMVp-lacZ) has been described previously (Ponnazhagan *et al.*, 1997a). The human adenovirus type 2 (Ad2) was provided by K.H. Fife (Indiana University School of Medicine, Indianapolis) and was propagated as previously described (Ponnazhagan *et al.*, 1994; Ponnazhagan *et al.*, 1997a).

Construction of recombinant plasmids and production of recombinant B19 virions:

The recombinant helper plasmids containing the B19-cap genes under the control of the CMV promoter were constructed by first cloning either the VP2 gene alone or VP1+VP2 genes isolated from the plasmid pYT-103 to yield plasmids pSP-37 and pSP-38, respectively. Subsequently, the portion of the B19-cap genes with the CMV promoter was cloned in the plasmid pAAV/Ad replacing the AAV-cap genes to yield plasmids pSP-42 and pSP-46, respectively. The recombinant helper plasmids pSP-42 and pSP-46 were used to package rescued and replicated recombinant AAV genomes containing the CMVp-lacZ gene sequences in 293 cells by the calcium phosphate transfection protocol as previously described (Ponnazhagan *et al., 1997a;* Ponnazhagan *et al., 1997b*). Harvesting and CsCl density gradient purification of the virus were done as described previously (Ponnazhagan *et al., 1997a;* Ponnazhagan *et al., 1997b;* Wang *et al.*, 1995). Quantitative slot blot analysis was performed to determine the physical titers of the virus stocks as previously described (Kube *et al.*, 1997; Srivastava and Lu, 1988; Srivastava *et al.*, 1989).

Electron Microscopy:

Recombinant B19-lacZ virions, purified on CsCl density gradients were stained with 3% phosphotungstic acid (pH 6.5) and visualized at a magnification of x80,000 using a Philips 400 electron microscope as previously described (Wang *et al.*, 1995).

Isolation of low-density and primitive progenitor human bone marrow cells and cellular differentiation:

Bone marrow aspirates were immediately diluted with an equal volume of Iscove's-modified Dulbecco's Medium (IMDM) containing 20 U/ml heparin. Low-density bone marrow (LDBM) cells were obtained by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density centrifugation. For the isolation of CD34+ cells, the mononuclear cells were labeled with anti-CD34 antibodies that were conjugated with magnetic particles. The labeled cells were passed through a magnetic separation column (Miltenyi Biotech, Sunnyvale, CA) and CD34- cells were allowed to flow through the column. CD34+ cells were subsequently eluted using MACS buffer (0.5% BSA and 5 mM EDTA in 1xPBS). The purity of the isolated CD34+ cells were determined by fluorescence-activated cell sorting (FACS) and ranged between 90-95%.

Differentiation of CD34+ cells into erythroid and myeloid lineages *in vitro* was achieved by the addition of 5 U/ml erythropoietin (Epo) and 10 ng/ml of granulocyte-colony stimulating factor (G-CSF), respectively, to the cells in liquid cultures supplemented with interleukin-3 (IL-3), interleukin-6 (IL-6) and stem cell factor (SCF) (Stem Cell Technologies, Vancouver, BC) as previously described.

Infection of human cells with recombinant AAV and B19 vectors and analysis of the transduced lacZ gene expression:

Human 293 and MB-02 cells, with or without erythroid-differentiation with Epo, were either mock-infected or infected at a particle to cell ratio of 200 at 37°C for 1 hr. Cells were washed and incubated in fresh medium at 37°C. Forty-eight hrs post-infection, cells were fixed and stained with the X-Gal substrate and examined under a Nikon inverted microscope for expression of the transduced lacZ gene as previously described (Ponnazhagan *et al.*, 1996). The LDBM and differentiated and undifferentiated CD34+ cells were either mock-infected or infected with the recombinant B19-lacZ or recombinant AAV-lacZ vectors at a particle to cell ratio of 200:1 and 100,000:1, respectively, at 37°C for 1 hr following which the cells were

grown in the presence of respective cytokines mentioned above for a period of 48 hrs. Analysis of expression of the transduced gene was performed using the FITC-conjugated substrate for β-Gal and the PE-conjugated substrates for CD34 and CD33 antigens or glycophorin. Briefly, cells were incubated with 300 mM chloroquine for 30 min. at 30°C following which chloroquine was removed by centrifugation and cells were incubated further with 33 mM Imagreen C12FDG β-Gal substrate (Molecular Probes Inc., Eugene, OR) for 30 min. at 37°C. Following centrifugation, the cells were resuspended in fresh culture medium and analyzed using a Beckton-Dickinson FACScanner as previously described (Ponnazhagan *et al.*, 1997a.

EXAMPLE 2

Recombinant AAV Genomes Encapsidated In Parvovirus B19 Capsids

The production of the recombinant B19 vectors was achieved by generating two helper plasmids, designated pSP-42 and pSP-46, by substituting the AAV-*cap* gene sequences in the AAV helper-plasmid pAAV/Ad (Samulski *et al.*, 1987) with the B19-*cap* gene sequences isolated from plasmid pYT103c (Srivastava *et al.*, 1989) leaving the rep gene sequences of AAV intact. The B19 sequences were cloned under the control of the CMV promoter. The recombinant helper-plasmid pSP-42 contains only the VP2 gene, and the plasmid pSP-46 contains both VP1+VP2 genes of the B19-*cap* sequences. Both helper-plasmids were initially tested for their ability to mediate efficient rescue and replication of the AAV genome from a recombinant AAV plasmid indicating functional trans-complementation by the AAV-*rep* proteins. The efficiency of rescue/replication of the recombinant AAV CMVp-*lacZ* genome by plasmids pSP-42 and pSP-46 was nearly the same as that by pAAV/Ad. The packaging of the recombinant B19-*lacZ* vectors was carried out by co-transfecting the pCMVp-*lacZ* plasmid with either pSP-42 or pSP-46 in 293 cells as described in Example 1. Recombinant AAV vector stocks containing the same CMVp-*lacZ* transgene were also prepared as previously described (Ponnazhagan *et al.*, 1996; Ponnazhagan *et al.*, 1997a). Based on quantitative DNA slot-blot analyses (Kube *et al.*, 1997), the recombinant B19 viral titers were determined to be approximately $1x10^8$ particles/ml when pSP-42 was used as a helper-plasmid. Interestingly, when pSP-46 was used as a helper-plasmid, the recombinant viral titers obtained were approximately $1x10^9$ particles/ml. Subsequently, the presence of viral particles exhibiting icosahedral structures of 25-30 nm diameter, similar to the size of parvoviral capsids, was also

confirmed by electron microscopy (FIG. 2). Taken together, these results suggested that the recombinant AAV genomes were indeed encapsidated in the B19 capsid structures.

EXAMPLE 3

Recombinant B19 virions fail to infect non-erythroid human cell lines

The recombinant B19-*lacZ* vector was tested for its ability to infect both 293 cells, known to be permissive for AAV infection, and undifferentiated or erythroid-differentiated MB-02 cells, known to be permissive for B19 infection and replication upon differentiation with Epo (Munshi *et al.*, 1993), but non-permissive for AAV infection (Ponnazhagan *et al.*, 1996). Both recombinant vector stocks were used at a virus particle-to-cell ratio of 200:1. Forty-eight hrs post infection, cells were analyzed for the transduced *lacZ* gene expression. These results are depicted in FIG. 3A-FIG. 3F. It is interesting to note that whereas the AAV-*lacZ* vector was readily able to transduce 293 cells as evidenced by the appearance of blue cells indicating expression of the transduced gene (FIG. 3B), there was no expression either in the mock-infected or the B19-*lacZ* vector-infected 293 cells (FIG. 3A and FIG. 3C). On the other hand, whereas there was no expression in either mock-infected (FIG. 3D) or AAV-*lacZ* vector-infected (Fig. 3, Panel E) MB-02 cells, as expected, the transgene expression could be detected in the B19-*lacZ* vector-infected MB-02 cells following erythroid-differentiation Munshi *et al.*, 1993 (FIG. 3F). The transduction efficiency also correlated with the extent of erythroid-differentiation (Ponnazhagan *et al.*, 1996). Undifferentiated MB-02 cells, which are non-permissive for B19 infection (Munshi *et al.*, 1993), could not be transduced by the B19-*lacZ* vector suggesting the ability of this vector to selectively transduce cells following erythroid-differentiation.

EXAMPLE 4

Recombinant B19 virions mediate high-efficiency, selective transduction of primary human hematopoietic progenitor cells in the erythroid lineage

The functional studies to determine erythroid cell targeting of the recombinant viruses were first performed using the recombinant virus containing the β-gal sequences. In each of the studies, the recombinant AAV-*lacZ* vector was also included as an appropriate control. Human low-density bone marrow (LDBM) cells, obtained from normal volunteers, were used

in the following three sets of studies. In the first set, LDBM cells were sorted for glycophorin-positivity to enrich the erythroid fraction. Approximately equivalent numbers of cells were either mock-infected or infected with the AAV-*lacZ* vector ($1x10^5$ particles/cell), or with the B19-*lacZ* vector (200 particles/cell) under identical conditions. Forty-eight hrs post-infection, cells were sorted with FITC-X-Gal and PE-glycophorin using a FACScanner to determine the percentage of cells expressing the *lacZ* gene. These results, shown in FIG. 4, indicated that even at a 500-fold lower viral titers, the recombinant B19 vector-mediated delivery of the transduced gene was significantly higher than that by the recombinant AAV vector in the erythroid fraction of primary human bone marrow cells which strongly suggested, but did not prove, the erythroid cell-specific targeting by the recombinant B19 vector.

In the second set of studies, $CD34^+$ cells from human LDBM were isolated and either mock-infected or infected with the recombinant AAV-*lacZ* or the B19-*lacZ* vectors at a particle to cell ratios of 100,000:1 and 200:1, respectively as described above. The transgene expression was analyzed 48 hrs post-infection using the FITC-X-Gal substrate and the PE-glycophorin marker as previously described. The results shown in FIG. 5 indicated specific targeting of the erythroid population of primitive progenitor cells by the B19 vector. The AAV vector, on the other hand, showed higher expression in the non-erythroid population of cells, an observation consistent with the inventors previously published studies (Wang *et al.*, 1995). The low transduction efficiency of the AAV vector in $CD34^+$ cells is also consistent with the inventors recent studies documenting a wide variation in the level of transduction by these vectors.

And finally, in the third set of studies, CD34+ cells were first differentiated into erythroid and myeloid lineages by treatments with Epo and G-CSF, respectively, for 10 days followed by either mock-infection or infection with the recombinant AAV-lacZ or the B19-lacZ vectors precisely as described above. Forty-eight hrs post-infection, cells were analyzed for the transgene expression using the FITC-X-Gal substrate and PE-glycophorin for the erythroid population, and with the FITC-X-Gal substrate and PE-CD33 for the myeloid population, respectively. These results shown in FIG. 6, once again indicated that the recombinant B19-

lacZ vector was highly efficient in selectively transducing the erythroid-differentiated population of CD34+ cells.

EXAMPLE 5

Human Endothelial Cell-Specific Transgene Expression Mediated By the Recombinant AAV-B19 Hybrid Vector

Since endothelial cells have been shown to be infected by parvovirus B19, the possibility of whether human umbilical vein endothelial cells (HUVEC) could be transduced by the recombinant AAV-B19 hybrid vector was tested.

Recombinant parvoviral vectors were constructed. These recombinant vectors that contained the bacterial β-galactosidase (*lacZ*) gene under the control of the human cytomegalovirus (CMV) immediate-early promoter within AAV inverted terminal repeat sequences encapsidated either in AAV capsids (vAAV-*lacZ*) or in B19 capsids (vB19-*lacZ*). Both recombinant vectors were purified by cesium chloride density centrifugation and used to transduce HUVEC under identical conditions.

Transduction of HUVEC with recombinant vB19-*lacZ* revealed a 5- to 9- fold higher transduction efficiency compared with recombinant vAAV-*lacZ* after 2 and 6 days, respectively.

These data indicate that human endothelial cells can be efficiently transduced by the AAV-B19 hybrid vector. These studies have important implications in the use of this hybrid vector in human gene therapy applications involving intravascular gene delivery.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically,

it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Anderson *et al.*, *Lancet i*, 1378, 1983.

Arap *et al.*, *Cancer Res.*, 55:1351-1354, 1995.

Berns and Bohenzky, *Adv. Virus Res.*, 32:243-307, 1987.

Berns and Giraud, *Curr. Top. Microbiol. Immunol.*, 218:1-23, 1996.

Berns, *Microbiol Rev.*, 54:316-329, 1990.

Bertran *et al.*, *J Virol.*, 70(10)6759-6766, 1996.

Broderick *et al.*, *Proc. Natl. Acad. Sci.*, 84:3349, 1987.

Brown *et al.*, *Lancet ii, 1033-1034, 1984.*

Burbage *et al.*, *Leuk Res*, 21(7):681-690, 1997.

Burgess and Kelly, *Annu Rev Cell Biol*, 3:243-293, 1987.

Bussemakers *et al.*, *Cancer Res.*, 52:2916-2922, 1992.

Caldas *et al.*, *Nat. Genet.*, 8:27-32, 1994.

Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977.

Casey *et al.*, *Oncogene*, 6:1791-1797, 1991.

Chavez *et al.*, *In: Methods in Cell Biology*, 43:263-288, Roth, M., Ed. New York, Academic Press, 1994.

Cheng *et al.*, *Cancer Res.*, 54:5547-5551, 1994.

Cheung *et al.*, *J Biol Chem.* 268(32):24303-10, 1993.

Cech *et al.*, *Cell*, 27:487-496, 1981.

Cotmore *et al.*, *Science*, 226:1161, 1984.

Couch *et al.*, *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Edelman and Crossin, *Annu. Rev. Biochem.*, 60:155-190, 1991.

Edelman, *Annu. Rev. Biochem.*, 54:135-169, 1985.

Enver *et al.*, *Proc. Natl. Acad. Sci.*, 86:7033, 1989.

Ferber *et al.*, *Mol Endocrinol.*, 5(3)319-326, 1991.

Forster and Symons, *Cell*, 49:211-220, 1987.

Forster and Symons, *Cell*, 49:211-220, 1987.

Freshner, "Animal Cell Culture: a Practical Approach", Second Edition, Oxford/New York, IRL Press, Oxford University Press, 1992.

Frixen *et al.*, *J. Cell Biol.*, 113:173-185, 1991.

Gerlach *et al.*, *Nature (London)*, 328:802-805, 1987

Gerlach *et al.*, *Nature*, 328:802-805, 1987.

Giancotti and Ruoslahti, *Cell*, 60:849-859, 1990.

Herman *et al.*, *Cancer Research*, 55(20):4525-4530, 1995.

Hollstein *et al.*, *Science*, 253:49-53, 1991.

Huang *et al.*, *Science*, 242:1563, 1988.

Hussussian *et al.*, *Nature Genetics*, 15-21, 1994.

Joyce, *Nature*, 338:217-244, 1989.

Joyce, *Nature*, 338:217-244, 1989.

Kamb *et al.*, *Nature Genetics*, 8:22-26, 1994.

Kamb *et al.*, *Science*, 2674:436-440, 1994.

Kearns *et al.*, *Gene Ther.*, 3:748-755, 1996.

Kim and Cook, *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.

Kim and Cook, *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.

Kotin and Berns, *Virol.*, 170:460-467, 1989.

Kotin *et al.*, *Genomics*, 10:831-834, 1991.

Kotin *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:2211-2215, 1990.

Kube *et al.*, *Nucl. Acids Res.*, 25:3375-3376, 1997.

Lawn *et al.*, *Cell*, 21:647, 1980.

Lee *et al.*, *Proc. Natl. Acad. Sci.*, 82:4360, 1985.

Lidor *et al.*, *Am J Obstet Gynecol*;177(3):579-585, 1997.

Liebhaber *et al.*, *Proc. Natl. Acad. Sci.*, 77:7054, 1980.

Lin and Guidotti, *J. Biol. Chem.*, 264:14408-14414, 1989.

Macejak and Sarnow, *Nature*, 353:90-94, 1991.

Mains *et al.*, *Mol. Endocrinol.*, 9:(1)3-13, 1995.

Massuda *et al.*, *Proc Natl Acad Sci U S A* , 94(26):14701-14706, 1997.

Matsura *et al.*, *Brit. J. Cancer*, 66:1122-1130, 1992.

Michaelis *et al.*, *Ann. Rev. Microbiol.*, 36:435, 1982

Michel and Westhof, *J. Mol. Biol.*, 216:585-610, 1990.

Michel and Westhof, *J. Mol. Biol.*, 216:585-610, 1990.

Morgan *et al.*, *Blood*, 78: 2860-2871, 1991.

Mori *et al.*, *Cancer Res.*, 54:3396-3397, 1994.

Munshi *et al.*, *J. Virol.*, 67:562-566, 1993.

Nobri *et al.*, *Nature*, 368:753-756, 1995.

Obrink, *BioEssays*, 13:227-233, 1991.

Odin and Obrink, *Exp. Cell Res.*, 171:1-15, 1987.

Okamoto *et al.*, *Proc. Nat'l Acad. Sci. USA*, 91:11045-11049, 1994.

Orlow *et al.*, *Cancer Res.*, 54:2848-2851, 1994.

Ostrove *et al.*, *Virology*, 113:532-533, 1981.

Ozawa *et al.*, *Blood*, 70:384-391, 1987.

Ozawa *et al.*, *Science*, 233:883-886, 1986.

Pelletier and Sonenberg, *Nature*, 334:320-325, 1988.

Ponnazhagan *et al.*, *Hum. Gene Ther.*, 8:275-284, 1997a.

Ponnazhagan *et al.*, *J. Gen. Virol.*, 77:1111-1122, 1996.

Ponnazhagan *et al.*, *J. Exp. Med.*, 179:733-738, 1994.

Ponnazhagan *et al.*, *J. Virol.*, 71:3098-3104, 1997b.

Racher *et al.*, *Biotechnology Techniques*, 9:169-174, 1995.

Reinhold-Hurek and Shub, *Nature*, 357:173-176, 1992.

Reinhold-Hurek and Shub, *Nature*, 357:173-176, 1992.

Renan, *Radiother. Oncol.*, 19:197-218, 1990.

Rouger *et al.*, *Rev. Fr. Transfers. Immun. Hematol*, 30:699, 1987.

Saarinen *et al*, *Blood*, 67:1411-1417, 1986.

Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

Samulski *et al.*, *EMBO J.*, 10:3941-3950, 1991.

Samulski *et al.*, *J. Virol.*, 61(10):3096-3101, 1987.

Sarver *et al.*, *Science*, 247:1222-1225, 1990.

Sarver *et al.*, *Science*, 247:1222-1225, 1990.

Scanlon *et al.*, *Proc Natl Acad Sci USA*, 88:10591-10595, 1991.

Scanlon *et al.*, *Proc Natl Acad Sci USA*, 88:10591-10595, 1991.

Serjeant *et al.*, *Lancet ii*, 595-597, 1981.

Serrano *et al.*, *Nature*, 366:704-707, 1993.

Serrano *et al.*, *Science*, 267:249-252, 1995.

Srivastava and Lu, *J. Virol*, 62:3059-3063, 1988.

Srivastava *et al.*, *J. Virol.*, 62:3059, 1988.

Srivastava *et al.*, *Cancer Genet Cytogenet.*, 35:(1)61-71, 1988.

Srivastava *et al.*, *J. Virol.*, 45:555-564, 1983.

Srivastava *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:8078-8082, 1989.

Srivastrava *et al.*, *Blood.* 76:1997-2004, 1990.

Summerford and Samulski, *J. Virol.*, 72:1438-1445, 1998.

Takahashi *et al.*, *Cancer Res.*, 52:734-736, 1992.

Top *et al.*, *J. Infect. Dis.*, 124:155-160, 1971.

Tratschin *et al.*, *Mol. Cell. Biol.*, 5:3251, 1985.

Umbas *et al.*, *Cancer Res.*, 52:5104-5109, 1992.

vondemBorne *et al.*, *Br. J. Haematol*, 63:35, 1986.

Wang *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:12416-12420, 1995.

Weinberg, *Science*, 254:1138-1145, 1991.

CLAIMS

1. An expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein said cassette is located between said inverted terminal repeats, said selected DNA sequence is operably linked to said promoter, and said vector lacks any AAV structural genes.

2. The vector of claim 1, wherein each of said inverted terminal repeats comprises nucleotides 1 to 125 of SEQ ID NO:1.

3. The vector of claim 1, wherein said promoter is selected from the group consisting of CMV IE, LTR, SV40 IE, HSV *tk*, β-actin, b19p6, human globin α, human globin β and human globin γ promoter.

4. The vector of claim 1, wherein said selected DNA sequence encodes a polypeptide.

5. The vector of claim 1, wherein said selected DNA encodes an antisense RNA.

6. The vector of claim 4, wherein said polypeptide is selected from the group consisting of a gene encoding alpha -globin, beta -globin, gamma -globin, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, neomycin resistance, luciferase, adenine phosphoribosyl transferase (APRT), retinoblastoma, insulin, mast cell growth factor, p53, p21, p16 and adenosine deaminase.

7. The vector of claim 5, wherein said antisense RNA is complementary to a segment of an oncogene.

8. The vector of claim 7, wherein said oncogene is selected from the group consisting of *myb*, *myc*, *ras*, *raf*, *erb*, *src*.

9. A B19 viral particle comprising an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein said cassette is located between said inverted terminal repeats, said selected DNA sequence is operably linked to said promoter, and said vector lacks any AAV structural genes.

10. A helper virus construct comprising two adenovirus inverted terminal repeats, an AAV *rep* gene and a B19 VP2 cap gene, wherein said *rep* and *cap* genes are under the control of at least one promoter and are located between said inverted terminal repeats.

11. The helper virus of claim 10, wherein said virus construct further comprises a B19 VP1 cap gene.

12. The helper virus of claim 10, wherein the VP2 gene is under the control of the CMV IE promoter.

13. The helper virus of claim 10, wherein said *rep* gene is under the control of the B19 p5 promoter.

14. A method for packaging an AAV expression vector comprising the steps of:

(i) providing an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein said cassette is located between said inverted terminal repeats, wherein said

selected DNA sequence is operably linked to said promoter, and said vector lacks any AAV structural genes;

(ii) providing a helper virus construct comprising two adenovirus inverted terminal repeats, an AAV *rep* gene and a B19 VP2 gene, wherein said *rep* and *cap* genes are under the control of at least one promoter and are located between said inverted terminal repeats;

(iii) contacting said expression vector and said helper virus construct with said host cell under conditions permitting the uptake of said expression vector and said helper virus construct by said cell;

(iv) infecting said transfected host cell with adenovirus; and

(v) harvesting B19 particles from said host cell.

15. The method of claim 14, wherein the helper construct further comprises a B19 VP1 gene.

16. The method of claim 14, wherein the multiplicity of infection of said adenovirus is about 10pfu.

17. The method of claim 14, wherein said contacting conditions comprise calcium phosphate precipitation, electroporation, microprojectile bombardment or lipofection.

18. The method of claim 14, wherein said harvesting comprises host cell disruption, virus isolation and heat inactivation.

19. The method of claim 18, wherein host cell disruption comprises freeze-thawing.

20. The method of claim 18, wherein virus isolation comprises a technique selected from the group consisting of centrifugation, filtration and ion exchange chromatography.

21. The method of claim 14, wherein said host cells are infected about 8 hours after transfer of said expression vector and said helper virus construct.

22. The method of claim 14, wherein said harvesting is performed between about 65 and about 72 hours after infection of said adenovirus.

23. A method for expressing a selected DNA sequence in a host cell comprising the steps of:

(i) providing a B19 viral particle comprising an expression vector comprising two adeno-associated virus (AAV) inverted terminal repeats and an expression cassette comprising a selected DNA sequence and a promoter active in eukaryotic cells, wherein said cassette is located between said inverted terminal repeats, wherein said selected DNA sequence is operably linked to said promoter, and said vector lacks any AAV structural genes;

(ii) contacting said viral particle with said host cell under conditions permitting infection of said host cell; and

(iii) culturing said host cell under conditions permitting the transcription of said DNA sequence.

24. The method of claim 23, wherein said DNA sequence encodes a polypeptide.

25. The method of claim 23, wherein said DNA sequence encodes an antisense mRNA.

26. The method of claim 23, wherein said host cell is an erythroid cell.

27. The method of claim 26, wherein said erythroid cell is a human erythroid cell.

28. The method of claim 23, wherein said host cell is selected from the group consisting of a bone marrow cell, a peripheral blood cell, an endothelial cell and myocardial cell.

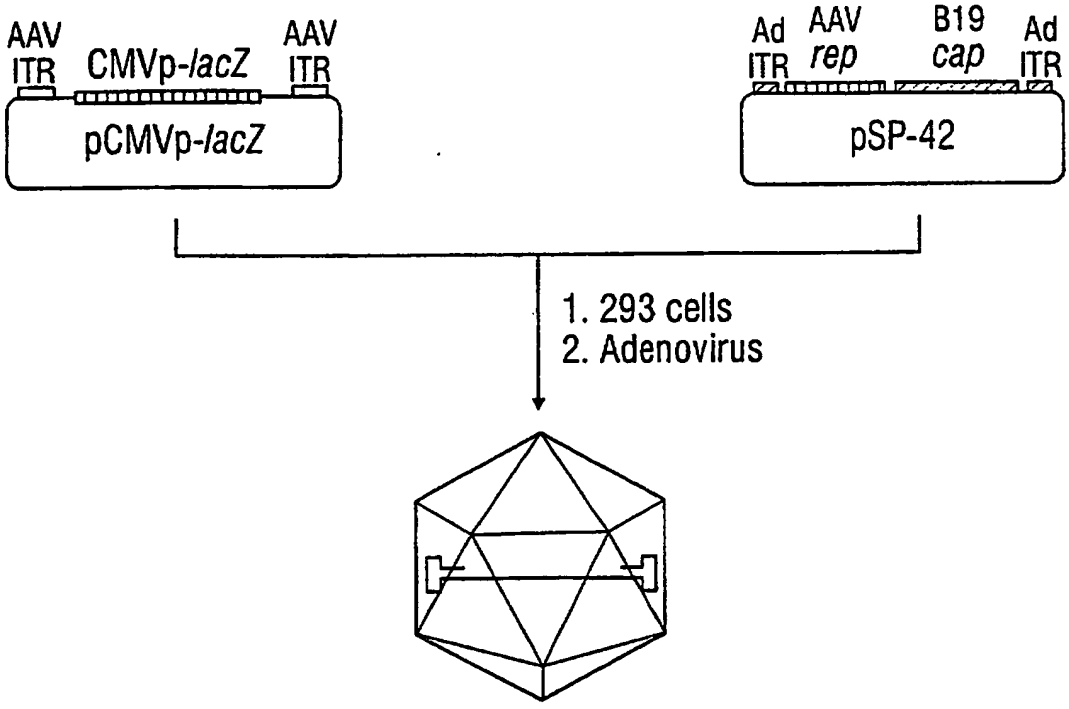

FIG. 1

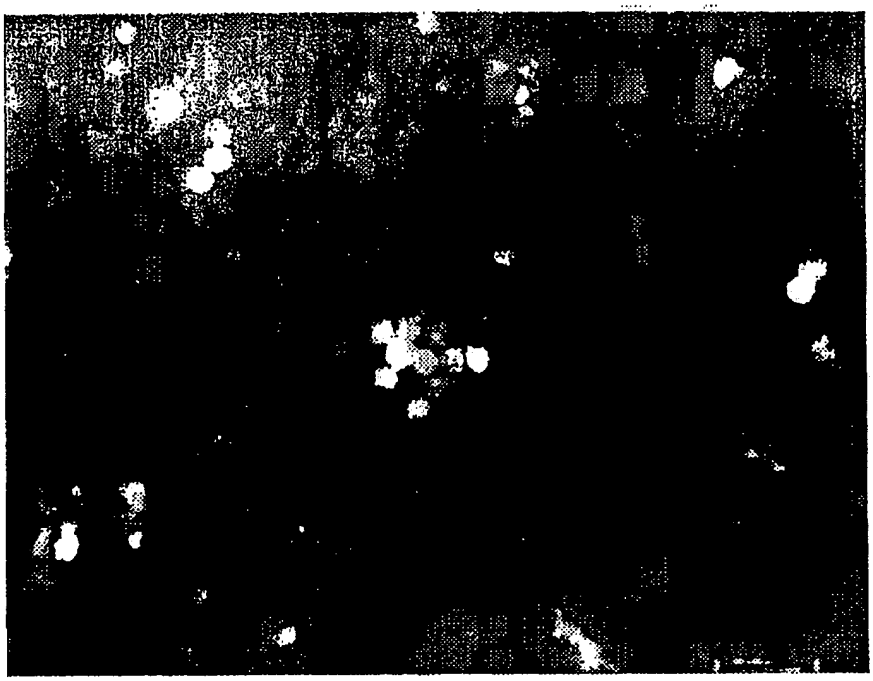

FIG.2

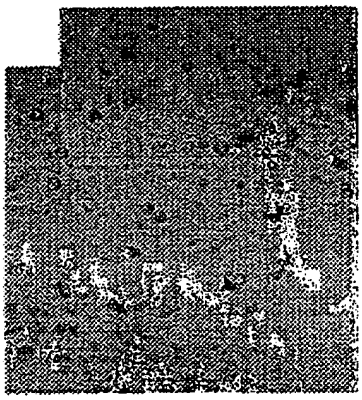

FIG.3A

FIG.3B

FIG.3C

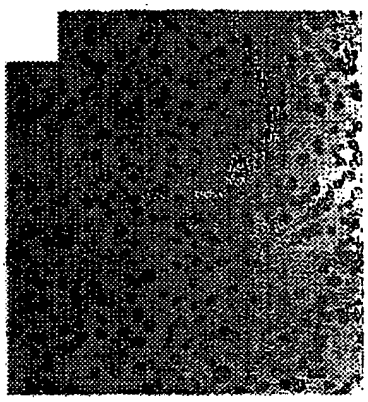

FIG.3D

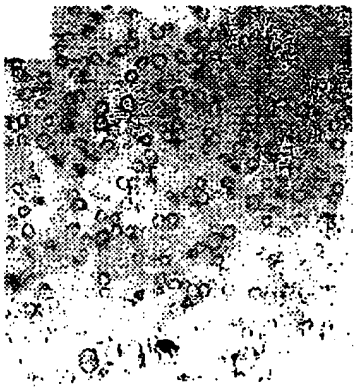

FIG.3E

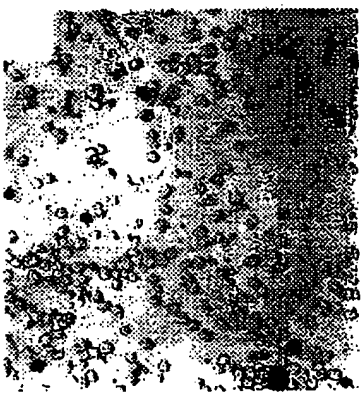

FIG.3F

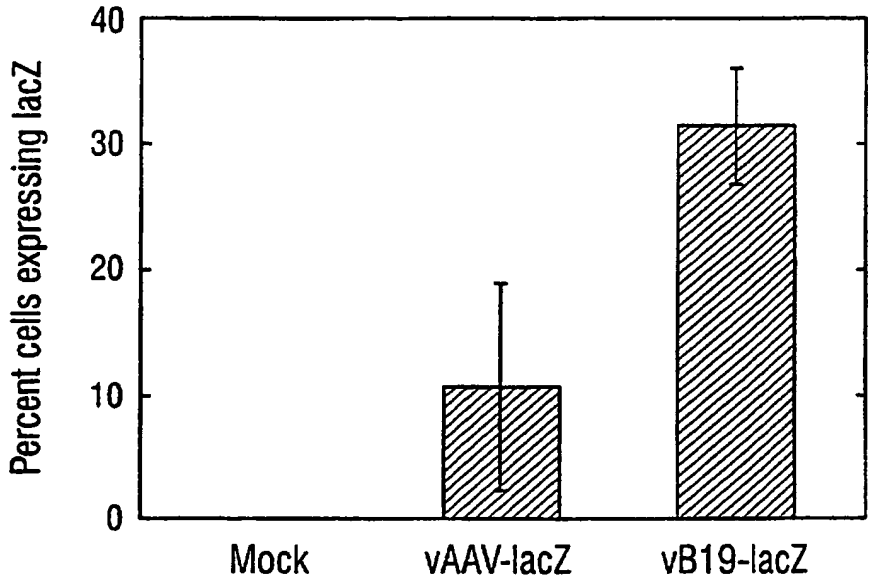

FIG. 4

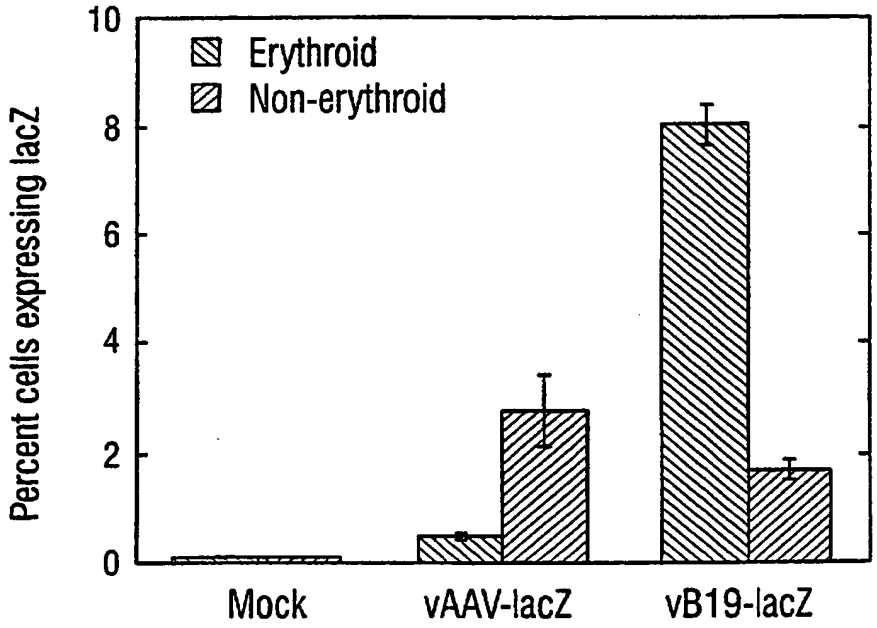

FIG. 5

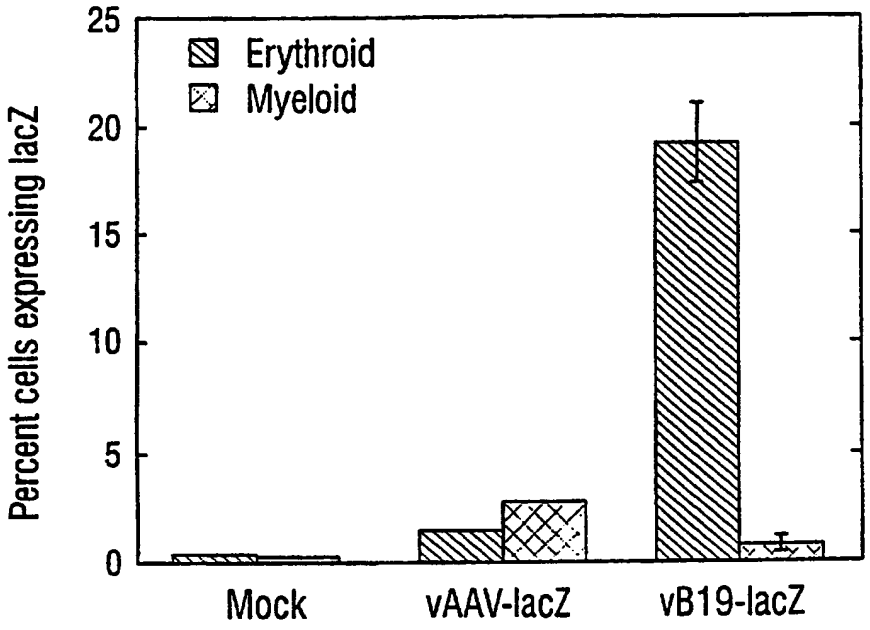

FIG. 6

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Advanced Research and Technology Institute
(B) STREET: 501 North Morton Street
(C) CITY: Bloomington
(D) STATE: IN
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 47404
(G) TELEPHONE: 512-418-3000
(H) TELEFAX: 512-474-7577

(ii) TITLE OF INVENTION: CHIMERIC PARVOVIRUS-BASED RECOMBINANT VECTOR SYSTEM THAT SPECIFICALLY TARGETS THE ERYTHROID LINEAGE

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: US Unknown
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/061,364
(B) FILING DATE: 08-OCT-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 145 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG CCGGGCGACC AAAGGTCGCC 60

CGACGCCCGG GCTTTGCCCG GGCGGCCTCA GTGAGCGAGC GAGCGCGCAG AGAGGGAGTG 120

GCCAACTCCA TCACTAGGGG TTCCT 145

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 225 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTTTAGCGGG CTTTTTTCCC GCCTTATGCA AATGGGCAGC CATTTTAAGT GTTTTACTAT 60

AATTTTATTG GTTAGTTTTG TAACGGTTAA AATGGGCGGA GCGTAGGCGG GGACTACAGT 120

ATATATAGCA CGGTACTGCC GCAGCTCTTT CTTTCTGGGC TGCTTTTTCC TGGACTTTCT 180

TGCTGTTTTT TGTGAGCTAA CTAACAGGTA TTTATACTAC TTGTT 225

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/21202

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N15/35 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 09239 A (RES CORP TECHNOLOGIES INC) 13 May 1993 see the whole document ---	1-9
X	SRIVASTAVA C H ET AL: "CONSTRUCTION OF A RECOMBINANT HUMAN PARVOVIRUS B19: ADENO-ASSOCIATED VIRUS 2 (AAV) DNA INVERTED TERMINAL REPEATS ARE FUNCTIONAL IN AN AAV-B19 HYBRID VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, no. 20, October 1989, pages 8078-8082, XP000608946 cited in the application see the whole document ---	1-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 1999	16/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/21202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 34670 A (CHILDRENS HOSPITAL INC) 21 December 1995 SEQ ID no.1 see claims 1-25; figure 3; example 1 ---	1-9
X	WO 94 13788 A (UNIV PITTSBURGH) 23 June 1994 see figures 1A,8.2. ---	1-9
X	WO 95 23867 A (LATTA MARTINE ;DENEFLE PATRICE (FR); PERRICAUDET MICHEL (FR); RHON) 8 September 1995 see page 4, line 23 - page 5, line 10; claims 1-32; figures 1-9; example 3 ---	1-9
X	PONNAZHAGAN S ET AL: "Differential expression in human cells from the p6 promoter of human parvovirus B19 following plasmid transfection and recombinant adeno-associated virus 2 (AAV) infection: human megakaryocytic leukaemia cells are non-permissive for AAV infection." JOURNAL OF GENERAL VIROLOGY, (1996 JUN) 77 (PT 6) 1111-22. JOURNAL CODE: I9B. ISSN: 0022-1317., XP002095110 see the whole document ---	1-9
A	S. WONG ET AL.: "Formation of empty B19 parvovirus capsids by the truncated minor capsid protein" J. VIROLOGY, vol. 68, no. 7, July 1994, pages 4690-4694, XP002095111 AM.SOC.MICROBIOL.,WASHINGTON,US see the whole document ---	1-28
A	N.C. MUNSHIL ET AL.: "Successful replication of Parvovirus B19 in the human megakaryotic leukemia cell line MB-02" J. VIROLOGY, vol. 67, no. 1, January 1993, pages 562-566, XP002095112 AM.SOC.MICROBIOL.,WASHINGTON,US see the whole document ---	1-28
A	US 5 508 186 A (SHIMADA TAKASHI ET AL) 16 April 1996 cited in the application see the whole document ---	1-28

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 98/21202

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	PONNAZHAGAN, S. ET AL: "Development of human parvovirus B19 vectors: Erythroid cell-specific delivery and expression of transduced genes." BLOOD, (NOV. 15, 1997) VOL. 90, NO. 10 SUPPL. 1 PART 1, PP. 602A. MEETING INFO.: 39TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY SAN DIEGO, CALIFORNIA, USA DECEMBER 5-9, 1997 THE AMERICAN SOCIETY OF HEMATOLOGY. ISSN: 0006-4971., XP002095113 Abstract no. 2674; ---	1-28
P,X	S. PONNAZHAGAN ET AL.: "Recombinant human parvovirus B19 vector: Erythroid cell-specific delivery and expression of transduced genes" J. VIROLOGY, vol. 72, no. 6, June 1998, pages 5224-5230, XP002095114 AM.SOC.MICROBIOL.,WASHINGTON,US see the whole document -----	1-28

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
PCT/US 98/21202

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9309239 A	13-05-1993	US 5252479 A	12-10-1993
		AU 657829 B	23-03-1995
		AU 3134093 A	07-06-1993
		CA 2098483 A	09-05-1993
		EP 0566732 A	27-10-1993
		JP 6504680 T	02-06-1994
		PT 101040 A	31-05-1994
WO 9534670 A	21-12-1995	US 5658785 A	19-08-1997
		AU 3124395 A	05-01-1996
		CA 2192215 A	21-12-1995
		EP 0764213 A	26-03-1997
		JP 10504185 T	28-04-1998
		US 5786211 A	28-07-1998
		US 5858775 A	12-01-1999
WO 9413788 A	23-06-1994	US 5478745 A	26-12-1995
		EP 0673415 A	27-09-1995
		JP 8504102 T	07-05-1996
		US 5869305 A	09-02-1999
WO 9523867 A	08-09-1995	FR 2716893 A	08-09-1995
		AU 1852695 A	18-09-1995
		CA 2184113 A	08-09-1995
		EP 0748385 A	18-12-1996
		JP 9509578 T	30-09-1997
		ZA 9501803 A	09-01-1996
US 5508186 A	16-04-1996	US 5827647 A	27-10-1998
		AU 631159 B	19-11-1992
		AU 4661389 A	12-06-1990
		CA 2002839 A	14-05-1990
		EP 0441897 A	21-08-1991
		JP 9313182 A	09-12-1997
		JP 2755817 B	25-05-1998
		JP 4502857 T	28-05-1992
		WO 9005538 A	31-05-1990